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Increased dietary fat contributes to dysregulation of the LKB1/AMPK pathway and increased damage in a mouse model of early stage ethanol-mediated steatosis

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Abstract

Objective—To examine the interaction of moderate and high dietary fat and ethanol with respect to formation of steatosis and regulation of the AMP-activated protein kinase (AMPK) pathway in a mouse model of chronic ethanol consumption.

Methods—Male C57BL/6J mice were pair-fed a modified Lieber-DeCarli diet composed of either moderate fat (30% fat-derived calories (MF)) or high fat (45% fat-derived calories (HF)) combined with increasing concentrations of ethanol (2–6%) for 6-weeks.

Results—Chronic ethanol consumption resulted in significant increases in plasma alanine aminotransferase in MF (1.84-fold) and HF mice (2.33-fold), yet liver triglycerides only increased significantly in the HF model (1.62-fold). Ethanol addition significantly increased plasma adiponectin under conditions of MF but not HF. In combination with MF, the addition of ethanol significantly decreased total and hepatic pThr^{172}AMPKα and acetyl CoA Carboxylase (ACC). HF plus ethanol decreased pSer^{108}AMPKβ yet a marked 1.5-fold increase in pThr^{172}AMPKα occurred. No change was evident in pSer^{79}ACC under conditions of ethanol and HF ingestion. In both models, nuclear levels of SREBP1c, ChREBP were decreased. Surprisingly, MF plus ethanol significantly elevated protein expression of MCAD, LCAD and VLCAD but did not significantly affect mRNA expression of other proteins involved in β-oxidation and fatty acid synthesis. HF plus ethanol significantly reduced mRNA expression of both SCD-1 and ELOVL5, but did not have an effect on MCAD or LCAD.

Conclusion—These data suggest that when co-ingested with ethanol, dietary fat differentially contributes to dysregulation of adiponectin-dependent activation of the AMPK pathway in the liver of mice.

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Keywords

alcohol; dietary fat; AMPK; hepatic steatosis

1. Introduction

Chronic alcoholic liver disease (ALD) is a significant contributor to liver based morbidity in humans. Steatosis is an early consequence of chronic alcohol consumption in mammals and continued drinking of excessive amounts of alcohol can subsequently lead to severe liver injury in the form of steatohepatitis, hepatic fibrosis and cirrhosis [1]. While the molecular mechanisms involved in the formation of alcohol induced steatosis remain to be elucidated, current research implicates cellular processes involved in regulating fatty acid metabolism including β-oxidation, fatty acid synthesis and lipid transport. The liver kinase B1 (LKB1)/ AMP-activated protein kinase (AMPK) regulatory pathway plays a critical role in fatty acid metabolism [2; 3]. Phosphorylation of AMPK by LKB1 increases the ability of AMPK to phosphorylate and inactivate a major rate-limiting enzyme in fatty acid synthesis, namely acetyl CoA carboxylase (ACC). ACC catalyzes the conversion of acetyl CoA to malonyl CoA [4] which is then utilized in the synthesis of saturated and unsaturated fatty acids. At high ratios of AMP/ATP, AMPK activity is increased and ACC activity is decreased leading to a decrease in cellular malonyl CoA. If AMP/ATP ratios are low, AMPK activity is decreased and excess malonyl CoA is used in the production of *de novo* fatty acid synthesis [5]. Fatty acid synthase (FASN) catalyzes the production of saturated fatty acids such as palmitate from malonyl CoA and palmitate is converted to stearate via fatty acid elongases such as ELOVL6 and desaturated via stearoyl CoA desaturase-1 (SCD-1) [6].

Previous reports of the effects of chronic ethanol on the regulation of AMPK and β-oxidation/fatty acid synthesis are conflicting. Early studies using rats indicated that chronic ethanol decreased the activity or expression of FASN and SCD-1 in the liver and ACC and FASN in adipose tissue [7; 8; 9]. In other studies, there was no difference in FASN or ACC activity suggesting that de novo fatty acid synthesis does not play a major role in ethanol induced fatty liver [10]. More recent studies have demonstrated equally opposing results. On one hand, in C57BL6 mice, chronic ethanol feeding decreased AMPK phosphorylation and activity and led to a corresponding increase in ACC activity, fatty acid synthesis and mRNA levels of SCD-1, ATP citrate lyase (ACLY) and FASN [11; 12; 13; 14]. Other studies have found either no change or an increase in AMPK activity promoting a decrease in fatty acid synthesis in mice [15; 16]. Saturated fat in combination with ethanol has been shown to have an effect on the AMPK pathway as well. In a comparison of the effects of ethanol and saturated or unsaturated high fat diets, 40% saturated fats were found to increase AMPK phosphorylation and ameliorated steatosis and liver damage in mice [17]. All of these studies used variable amounts of fat, ethanol concentration and length of ethanol feeding.

Recent reports have suggested that the daily recommended allowance for dietary fat in humans is approximately 25–35% of total caloric intake. In the present study, we examined the effect of unsaturated dietary fat and ethanol on the AMPK pathway and expression of enzymes involved in fatty acid synthesis was examined. Mice were fed an ethanol-containing diet with either a moderate fat (30%) or a high fat (45%) diet for 6 weeks. We report that the addition of increased dietary fat leads to an increase in liver damage in conjunction with steatosis and contributes to dysregulation of AMPK signaling. Furthermore, under both dietary conditions, an overall decrease in expression of enzymes involved in fatty acid synthesis occurs following chronic ethanol consumption.

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2. Materials and methods

2.1 Animal Model and dietary information

C57BL/6J male mice (The Jackson Laboratory, Bar Harbor, ME), 6–8 weeks of age in groups of 12 were fed a modified Leiber-DeCarli diet (30% fat-derived calories (MF) or 45% fat-derived calories (HF) (Table 1)) (Bio-Serv, Frenchtown, NJ) consisting of isocaloric control and ethanol-treated animals [18]. Ethanol content was increased each week starting with 2% (v/v) ethanol, with the ethanol-derived calories increasing by 1% on a weekly basis until sacrifice; week 6 consisted of 6 % ethanol (v/v) or 31.8% ethanol-derived calories. In the control animals, calories derived from ethanol were replaced isocalorically by carbohydrates in the form of maltodextrin. Food consumption was monitored daily and body weights were measured once per week. Upon completion of the study, animals were anesthetized via intraperitoneal injection with sodium pentobarbital and euthanized by exsanguination. Blood was collected from the inferior vena cava and plasma separated via centrifugation at 4°C and assayed for alanine aminotransferase (ALT) activity (Sekisui Diagnostics, P.E.I., Canada). Excised livers were weighed and subcellular fractions obtained via differential centrifugation as previously described (6). All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Colorado and were performed in accordance with published National Institutes of Health guidelines.

2.2 Western Blotting

Proteins from either whole liver extracts or subcellular fractions were subjected to standard SDS-PAGE and transferred to PVDF (GE Healthcare, Picataway, NJ). Membranes were blocked for 60 minutes with a tris-buffered saline solution containing 1% Tween-20 (TBST) and 5% non-fat dry milk and probed overnight with primary antibodies directed against $pSer^{428}$LKB1, $pThr^{172}$AMPKα, $pSer^{108}$AMPKβ, $pSer^{79}$ACC, total LKB1, total AMPKα, total AMPKβ, total ACC (All from Cell Signaling, Danvers, MA), FASN (Pierce-Thermofisher, Rockford, IL), SCD-1 (Santa Cruz Biotechnology, Santa Cruz, CA), ELOVL5 (ABCAM, Cambridge, MA), ACLY (ABCAM), β-actin (Sigma Aldrich, St. Louis, MO). A horseradish peroxidase conjugated secondary (Jackson ImmunoResearch Inc. West Grove, PA) was then applied and membranes developed using ECL-Plus Reagent (GE Healthcare). Chemiluminescence was visualized using either film or a Storm 860 scanner from Molecular Dynamics (Sunnyvale, CA).

2.3 Triglycerides

Liver triglycerides were extracted from whole liver homogenates using chloroform: methanol (2:1) and measured using a kit from Sigma-Aldrich according to the manufacturer’s protocol. All values were normalized against tissue weight.

2.4 Histological analysis

Sections of freshly excised liver tissue were placed in 10% neutral buffered formalin (Sigma-Aldrich) for 16 hours, washed in 70% ethanol for 1 hr followed by incubation in 70% ethanol overnight. Samples were then processed, embedded in paraffin and mounted on slides by the UC Denver Histology Core. Hematoxylin and Eosin staining was performed by the UC Denver Histology Core. IHC was performed using a rabbit polyclonal antibody directed against CYP2E1 (Millipore, Billerica, MA) as previously described (7). Images of H&E stained or IHC stained liver sections were captured on an Olympus BX51 microscope equipped with a four megapixel Macrofire© digital camera (Optronics, Goleta, CA) using the PictureFrame© Application 2.3 (Optronics, Goleta, CA).

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2.5 Serum Adiponectin

Serum isolated from each group was diluted 1:1000 and examined for adiponectin via ELISA according to the manufacturer's instructions (Millipore, Billerica, MA). Absorbances for each sample were read at 450nm and 595nm using a microtiter plate reader (Molecular Devices, San Diego, CA) and

2.6 Statistical Analysis

Relative densitometry of Western blots was quantified using ImageJ (http://rsb.info.nih.gov/ij/). All data and statistical analysis was performed using Graph Pad Prism 4.02 for Windows (GraphPad Software, San Diego, CA). When comparing moderate fat control and ethanol samples with high fat control and ethanol samples a two-way analysis of variance followed by Bonferroni *post hoc* correction was utilized. All data are expressed as mean ± S.E.M. and p values <0.05 were considered significant.

3. Results

3.1 Effects of increased dietary fat and ethanol on liver weight and liver to body weight ratio

The data presented in Table 2 describes the effect of the four feeding regimens on body weight, overall liver weight and liver/body weight ratio. Comparing the MF and the MF+E groups, chronic ethanol resulted in a 1.91-fold decrease in total weight gain in the MF animals. Comparing the HF and HF+E animals, the increase in dietary fat in conjunction with ethanol led to a 3.17-fold decrease in total weight gain. Overall, HF+E animals gained less weight than their MF+E counterparts. In addition, the HF control animals consumed more and gained more than the MF control animals. Comparing the average daily energy intake, in the moderate fat and the high fat groups, the ethanol-fed animals consumed slightly higher total kcal compared to controls. There were no significant differences in ethanol consumption between groups (Table 2). Examining the overall change in liver to body weight ratio, only the HF+E exhibited a statistically significant −1.15-fold change in overall liver to body weight ratio compared to their respective controls.

3.2 Biochemical characterization of animals fed MF+E or HF+E diet

The effects of MF+E or HF+E on serum ethanol concentrations, ALT and liver triglycerides, were examined (Table 3). Both MF+E and HF+E animals had significantly higher ALT values than their respective controls. Compared to MF plus ethanol, the addition of HF plus ethanol led to a greater increase in ALT values (MF+E 1.84-fold, HF+E 2.33-fold. Using 2-way ANOVA, there was no significant interaction between the increased addition of dietary fat and ethanol. Compared to isocaloric controls, only the addition of a high fat diet plus ethanol led to a significant increase in liver triglycerides (HF+E 1.62-fold). Once again however, a significant interaction between increased dietary fat and ethanol was not evident. The effect of dietary fat on ethanol metabolism as measured by serum ethanol concentrations was assessed. From the data in Table 3, changes in dietary fat did not impact blood ethanol concentrations.

3.3 Hematoxylin and Eosin staining show large cytoplasmic lipid droplets in both the MF+E and HF+E livers

Corresponding to an increase in liver triglycerides, hematoxylin and eosin staining of liver sections presented in Figure 1 indicate an increase in macrosteatosis in both MF+E and HF+E liver sections when compared to sections from their respective controls. Steatosis was predominantly located in zone 2 with minor accumulation of lipid droplets in zone 1.

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3.4 Induction of CYP2E1 in MF+E and HF+E livers

The ingestion of ethanol is well documented to induce hepatic Cytochrome 450 2E1 [19; 20]. To evaluate the extent of CYP2E1 induction, IHC was performed on tissue sections from MF+E and HF+E mice. The data presented in Figure 2A demonstrate substantial induction of CYP2E1 visible in=both the MF+E and HF+E mice. The MF+E animals exhibited slightly less intense staining than the HF+E animals. To validate these data, a Western blot was performed. As shown in Figure 2B and 2C, CYP2E1 expression is increased in the MF+E and HF+E groups. Subsequent quantification of CYP2E1 expression demonstrated a 2-fold increase in livers from MF+E and a 4-fold increase in the HF+E mice compared to pair-fed controls, which is consistent with the immunohistochemistry data. Two-way ANOVA indicated a significant interaction between dietary fat and ethanol in the induction of CYP2E1.

3.5 Dysregulation of AMPK signaling by dietary fat and ethanol in mice

The LKB1/AMPK pathway contributes to regulation of metabolic pathways in the liver. The effects of dietary fat on LKB1 and its downstream signaling intermediates were analyzed via total protein expression and phosphorylation of LKB1, AMPKα/β and ACC (Figure 3A and 3B). Compared to isocaloric controls, MF+E led to a decrease in total expression of LKB1, AMPKα and ACC. Increasing dietary fat content led to no significant change in expression of LKB1, AMPKα or ACC when compared to the respective HF control animals. The LKB1/AMPK pathway is regulated by phosphorylation. As shown in Figure 3C and 3D, $pSer^{428}$LKB1 increased significantly in the MF+E mice and decreased significantly in HF +E mice. The immediate substrate of LKB1 is the heterotrimer AMPKα/β/γ [21]. In MF+E animals, levels of $pThr^{172}$AMPKα significantly decreased. The addition of HF+E led to a significant increase in $pThr^{172}$AMPKα but surprisingly, $pSer^{108}$AMPKβ significantly decreased. AMPK phosphorylates Ser^{79}ACC thereby inhibiting its activity and promoting β-oxidation. Phosphorylation of ACC significantly decreased in the MF+E but not the HF+E mice. Utilizing two-way ANOVA, a significant interaction was identified between dietary fat and ethanol for both $pSer^{428}$LKB1 and $pThr^{172}$AMPKα.

Serum adiponectin has been identified as an extracellular regulator of AMPK. Increased serum adiponectin correlates with increased AMPK phosphorylation and activation [22]. From Figure 3E, serum adiponectin was significantly increased in the MF+E animals. Ethanol did not have a significantly effect on adiponectin in the HF+E model. Using 2-way ANOVA, a significant interaction was present between increasing dietary fat but not with the addition of ethanol. Given that serum adiponectin was increased under MF+E conditions, relative mRNA expression was determined using a limited microarray analysis (Supplementary Table 1). From the array, there were no significant differences in hepatic mRNA expression of *Acrp30*, *ADIPOR1*, *ADIPOR2* or *PNPLA2* in either model.

3.6. Chronic ethanol-induced changes in mitochondrial acyl-CoA dehydrogenases in MF and HF mice

In other animal models, increases in AMPK phosphorylation have been implicated in increased expression of enzymes involved in mitochondrial β-oxidation. As described above, AMPK can promote β-oxidation by phosphorylating Ser^{79} of ACC and inhibiting its activity and phosphorylation of ACC significantly decreased in the MF+E but not the HF+E mice. In order to further characterize the effects of dietary fat in ALD we examined the effects of MF+E and HF+E on mitochondrial fatty acyl-CoA dehydrogenase (VLCAD, MCAD, LCAD, SCAD) expression by Western blotting. Compared to isocaloric controls, MF+E led to a significant increase in VLCAD, LCAD and MCAD. In the HF+E animals, only VLCAD was significantly elevated.

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3.7 Chronic ethanol induced changes in nuclear ChREBP and SREBP1 in HF and MF mice

Nuclear localization of both SREBP1c and ChREBP are involved in regulation of *de novo* lipogenesis [6]. AMPK has been implicated in the regulation of both SREBP1 and ChREBP. Phosphorylation of ChREBP by AMPK prevents nuclear translocation [23]. Nuclear localization of SREBP1c and ChREBP were examined in both the MF and HF models. As shown in Figures 5A–C, compared to isocaloric controls, levels of nuclear SREBP1c trended down in MF+E and significantly decreased in HF+E mice. Nuclear localization of ChREBP significantly decreased in MF+E mice and trended down in HF+E mice. Corresponding to the decrease in ChREBP nuclear localization, cytosolic levels of ChREBP were increased in both models.

3.7 Chronic ethanol decreases expression of enzymes involved in de novo fatty acid synthesis in MF and HF mice

Decreased levels of nuclear SREBP and ChREBP have been demonstrated to result in decreased fatty acid synthesis. In addition, dephosphorylation of ACC promotes fatty acid synthesis. Overall protein expression of key enzymes in fatty acid synthesis ACLY, FASN, ELOVL5 and SCD-1 were examined by Western blotting. Consistent with the reduced nuclear localization of SREBP and ChREBP, quantification of the data in Figure 6A and 6B demonstrate that addition of ethanol reduced expression of ACLY and SCD-1 in both the moderate fat and the high fat groups (Figure 6C). Utilizing 2-way ANOVA, SCD-1 indicated a significant interaction with dietary fat and ethanol on expression.

3.8 mRNA expression of proteins regulated by AMPK signaling

Activation or inactivation of AMPK can result in increased or decreased expression of proteins involved in both *de novo* lipogenesis, fatty acid transport and β-oxidation. To provide additional data with respect to AMPK signaling a limited microarray was performed using an arbitrary 2-fold cut-off in gene induction/repression [24]. As shown in Supplementary Table 1, examining *de novo* lipogenesis, in the MF+E fed animals there were no significant changes in mRNA expression in *FAS, GPAT1, SCD-1, ME, ELOVL2,3,5* or *6*. In the HF+E animal, chronic ethanol suppressed expression of *SCD-1* (−3.51 fold), *ELOVL5* (−2.00 fold) and *ELOVL3* (−6.51 fold). All other proteins involved in *de novo* lipogenesis did not significantly differ from those observed in the controls. Examining β-oxidation, surprisingly, the MF+E animals did not exhibit significant differences in *CPT-1α*, *ACOX1*, *MCAD*, *SCAD*, *LCAD*, *PPARα*, or *PGC1α* mRNA expression. In the HF+E animals, only *PPARα* (−2.53 fold) exhibited a significant change in mRNA expression. Given that in both models there is increased lipid accumulation that may affect lipid transport expression of fatty acid transport genes was examined. From Supplementary Table 1, there were no significant differences in either model in mRNA expression of *CD36* or *MTTP*.

4. Discussion

Depending on the amount of alcohol consumed, approximately 90% of chronic alcoholics will develop fatty liver or steatosis as an early marker of liver damage [25]. Of these, 10–35% will progress from simple steatosis to steatohepatitis and 5–15% will develop fibrosis. The biochemical mechanisms which lead to steatosis in ALD are not fully characterized but it is clear that multiple factors contribute to disease progression [26]. These include increased lipid peroxidation, oxidative stress and dysregulation of lipid metabolism [2; 27; 28; 29]. In this study, the effects of chronic ethanol feeding combined with either 30% or 45% dietary fat was examined. Consistent with previous reports, increasing the total amount of dietary fat significantly increased the amount of hepatic triglycerides and hepatocellular

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damage as determined by ALT [30]. Surprisingly, in the present study there is not a significant difference when the moderate fat groups are compared to the high fat groups.

In ALD, CYP2E1 is involved in the detoxification of ethanol [18; 31]. Previous reports have suggested an effect of dietary fat and CYP2E1 expression in rats [30; 32]. The addition of dietary medium-chain triglycerides instead of long chain triglycerides led to a decrease in overall liver damage and CYP2E1 expression [32]. In the present study, the relative composition of fatty acids remained the same (primarily long chain) and the total amount of long chain fatty acids was replaced with carbohydrates in the form of maltodextrin (MF+E). Further, MF+E exhibited less induction of CYP2E1, reduced triglyceride accumulation and hepatocellular damage when compared to HF+E. This is in agreement with previous studies that indicated that decreasing dietary fat along with increased carbohydrates resulted in reduced ALT, reduced triglyceride accumulation and reduced CYP2E1 induction [33]. During ALD, CYP2E1 induction occurs primarily in the perivenous region [31; 34]. In this study, tissue examination of the MF+E and HF+E mice revealed less intense staining of CYP2E1 in the MF+E animals when compared to HF+E animals. Concurrently, MF+E exhibited less reduction of CYP2E1 expression in the periportal region.

Cyclic AMP activated kinase is activated under conditions of low energy and its corresponding AMP/ATP ratio. Alternatively, increased serum adiponectin results in an increase in AMPK activation via adiponectin receptors 1 and 2 [22]. In the literature, reports concerning the effects of ethanol on activation of the AMPK pathway in mice vary depending on the amount of ethanol and the duration of feeding. In some studies, AMPK phosphorylation is increased whereas in others, AMPK phosphorylation is decreased [12; 14; 15; 35]. In one report, 40% saturated fat plus ethanol resulted in a 2-fold increase in AMPKα phosphorylation. Concurrently, 40% PUFA plus ethanol resulted in a slight decrease in AMPKα phosphorylation [17]. This further substantiates the role that dietary fat plays in regulation of AMPK by ethanol. In the present study, adiponectin levels are elevated in the MF+E model but this did not correlate with increased mRNA expression of ADIPOR1/2 or translate to an increase in pThr^{172}AMPK and pSer^{79}ACC. This has been demonstrated in another model that utilized hypertensive rats. In that model, increased hypertension led to elevated liver triglycerides and adiponectin but phosphorylation of AMPKα and ACC were decreased [36]. Feeding mice a high fat diet alone is known to reduce adiponectin and AMPK phosphorylation [37; 38]. In our model, HF+E did not result in an increase in adiponectin but there is an increase in AMPKα phosphorylation. Surprisingly this did not correspond to an increase in phosphorylation of AMPKβ and ACC. Clearly, in our model, the concomitant presence of high dietary fat with ethanol results in dysregulation of AMPK signaling. This effect has been previously identified in an overfeeding/obesity intragastric model, whereby AMPK phosphorylation was induced by ethanol yet ACC phosphorylation was decreased [15]. In that study, the authors did not examine AMPKβ phosphorylation. Phosphorylation of AMPKβ is required for full activation of the AMPK heterotrimer [39].

The effects of chronic ethanol administration on phosphorylation of LKB1 have not previously been examined. In this study, LKB1 phosphorylation remains decreased in both the MF+E and HF+E model whereas expression is only decreased in the MF+E model. One hypothesis is that phosphorylation of LKB1 at Ser^{428} is required for activation and subsequent AMPKα phosphorylation at Thr^{172} [40]. Other research has suggested that activation of LKB1 is independent of Ser^{428} phosphorylation [41]. Thus, based on our results, in the HF+E mice, the increase in AMPK phosphorylation may be independent of LKB1 phosphorylation.

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In the liver, both ChREBP and SREBP regulate hepatic lipogenesis. Both transcription factors bind to promoters and positively affect transcription of lipogenic proteins such as ACC, FASN, SCD-1 and ELOVL6 [6; 42; 43; 44; 45; 46]. ChREBP is in part regulated by the ability of AMPKα to phosphorylate Ser^{568}. Dephosphorylation results in increased nuclear localization of ChREBP [23; 47]. The decrease in AMPK downstream signaling may provide a mechanism for decreased ChREBP nuclear localization in MF+E and HF+E models.

In mouse models of nonalcoholic fatty liver disease, AMPKα is a negative regulator of SREBP1c. Increased AMPKα phosphorylation typically results in a decrease in nuclear SREBP1c. In both the intragastric model and the NAFLD human patient study, levels of SREBP1c were increased [3; 15]. This suggests an additional level of regulation between AMPKα/ACC and SREBP1c. Other studies however clearly indicate no change in SREBP following chronic ethanol in rats [48]. In the present study, levels of SREBP1c trend down in both MF+E and HF+E treated mice indicating dysregulation of signaling between AMPK and SREBP. Corresponding to the decrease in SREBP1c, nuclear localization of ChREBP is also decreased in HF+E and MF+E mice.

In the ALD literature, there are also opposing results with respect to activity and mRNA expression and protein expression of lipogenic proteins. Early studies demonstrated a decrease in SCD-1 and FASN enzymatic activities following chronic ethanol consumption in rats [7; 8]. More recently, Northern blotting and mRNA expression indicated ethanol-mediated increases in *SREBP1c*, *Scd1*, *FASN*, *ACLY*, *ELOVL2, ELOVL3* and *ACC* expression [11; 13; 49]. These studies were all for 4 weeks and utilized either a 10% low fat diet (cocoa butter/safflower oil) in combination with ethanol or dietary fat content was not stated. The 10% low fat studies also found a substantial decrease in AMPKα phosphorylation. In the MF+E animals, AMPKα phosphorylation is reduced but not to the levels demonstrated by You et al.[12]. It may be that further decreasing dietary fat content may result in lower AMPKα activation. In summary, the discrepancy between previous low fat reports and our data may be due the length of ethanol administration, the quantity and source of dietary fat or a combination of both.

Examining the high fat model, another report using a 6-week chronic ethanol feeding regimen and the Lieber DeCarli diet suggests either no significant change (*SREBP1c, FASN, ACC*) or downregulation of mRNA expression (*Scd1, ELOVL5*) following chronic ethanol administration [50]. We also find that chronic ethanol feeding utilizing a similar feeding protocol, *Scd-1* and *ELOVL5* are suppressed. Thus increasing dietary fat content in combination with length of ethanol administration can lead to changes in expression of *de novo* lipogenesis (DNL) enzymes. The decrease in DNL expression in this study does not explain why there are increased levels of steatosis and triglycerides. Investigators have previously suggested that there are different pools of fatty acid CoA within hepatocytes and that ethanol can differentially regulate these pools [50]. Based on our data and others, it is possible that early steatosis formed during chronic ALD under high fat conditions is not due to *de novo* fatty acid synthesis or changes in expression of β-oxidation but due to alterations of other pathways [10; 50; 51]. There is data to support this hypothesis. Several studies have implicated defective VLDL secretion as a contributing factor in ethanol-induced steatosis [52]. In rats, chronic ethanol feeding resulted in decreased perivenous VLDL secretion [53]. The addition of betaine increased VLDL production and attenuated the development of alcoholic steatosis [54].

In summary, when compared to a moderate fat diet, the addition of a high fat diet plus ethanol leads to significant increases in liver damage, triglycerides and CYP2E1 expression. However, a high fat diet subtlety changes regulation of AMPK. As displayed in Figure 7, the

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increase in dietary fat may contribute to an increase in AMPKα phosphorylation but there is dysregulation of AMPK downstream signaling as shown by the lack of increased phosphorylation of ACC. This corresponds to no significant changes in mRNA of β-oxidation dependent genes. In the moderate fat animals, addition of ethanol resulted in increased serum adiponectin but phosphorylation of AMPKα did not increase. This suggests that regulation of adiponectin signaling may be defective under conditions of moderate fat plus ethanol. Both models display decreased nuclear localization of SREBP and ChREBP resulting in downregulation of fatty acid synthetic enzymes. Thus, depending on dietary fat content, ethanol may dysregulate hepatocyte signaling on different levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACC	acetyl CoA carboxylase
ALD	alcoholic liver disease
ALT	alanine amino transferase
AMPK	AMP-activated protein kinase
ACLY	ATP citrate lyase
ChREBP	carbohydrate response element binding protein
CYP2E1	cytochrome p450 2E1
ELOVL5	fatty acid elongase 5
ELOVL6	fatty acid elongase 6
FASN	fatty acid synthase
LKB1	liver kinase B1
LCAD	long chain acyl-CoA dehydrogenase
MCAD	medium chain acyl-CoA dehydrogenase
ME	malic enzyme
PP2A	protein phosphatase 2A
SCAD	short chain acyl-CoA dehydrogenase
SREBP	sterol response element binding protein 1
SCD-1	stearoyl CoA desaturase 1
VLCAD	very long chain acyl CoA dehydrogenase

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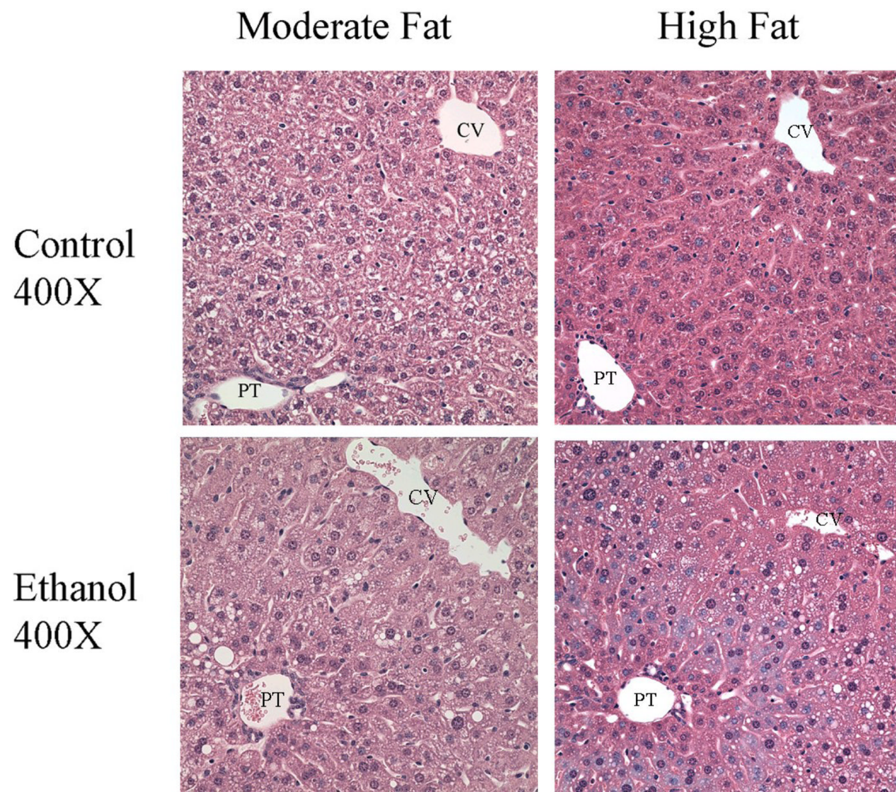

Fig. 1.
Increased steatosis in zone 2 in MF+E and HF+E mice. Hematoxylin and eosin staining of tissue sections from MF+E and HF+E mice. (CV, central vein, PT, portal triad). Original Magnification 400X.

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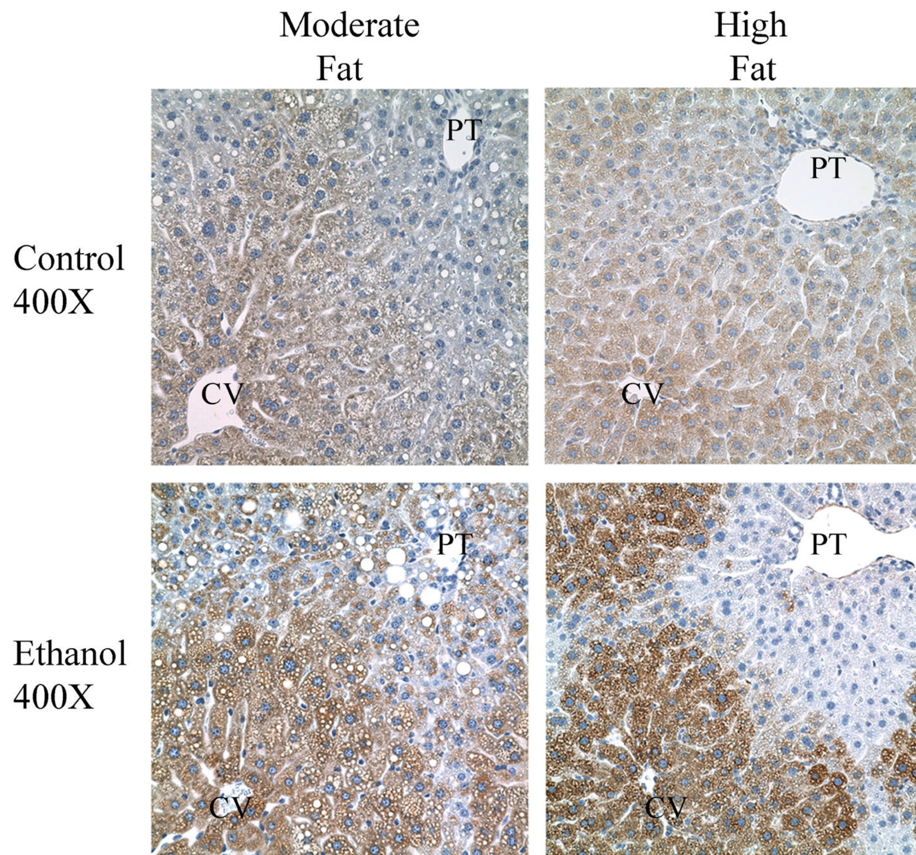
Figure 2A



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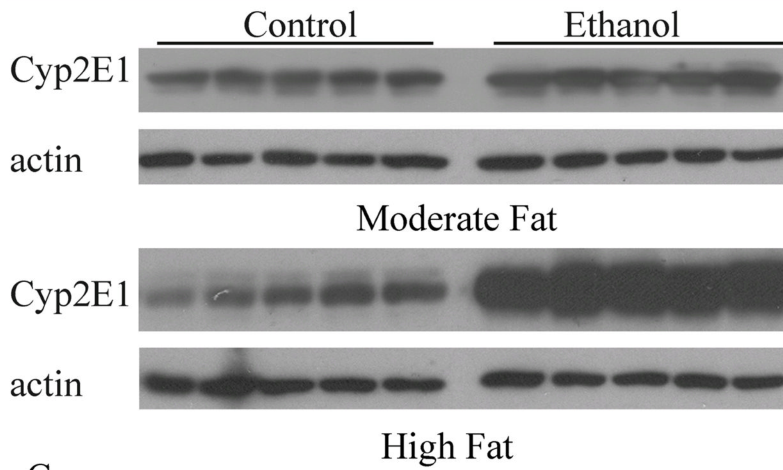

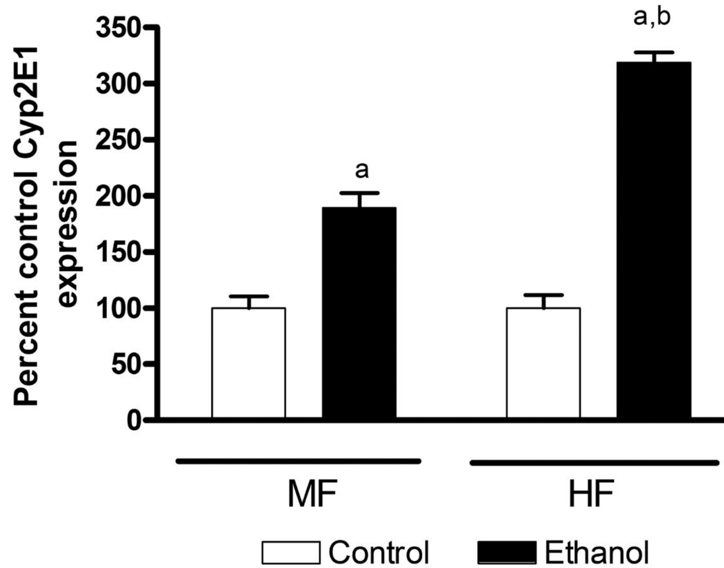

Fig. 2.

Chronic ethanol leads to increased CYP2E1 in MF+E and HF+E mice (A) Immunohistochemical analysis of CYP2E1 expression in MF, MF+E, HF and HF+E mice. Note positive staining in centrilobular region (zone 3) in both ethanol feeding models. (CV, central vein; PT, portal triad) Magnification 400X. (B) Western blotting analysis of CYP2E1 expression in MF, MF+E, HF and HF+E mice. (C) Quantification of the Western blots presented in Figure 2B (actin normalized). Data are means± SEM as analyzed by two-way ANOVA with a Bonferroni *post hoc* analysis (MF±E compared to HF±E groups). Means without a common superscript letter are significantly different (N=6 mice/group ($p<0.05$)).

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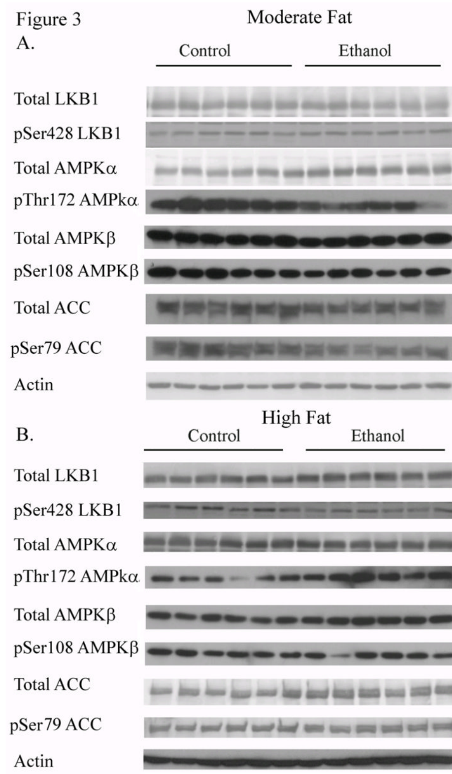

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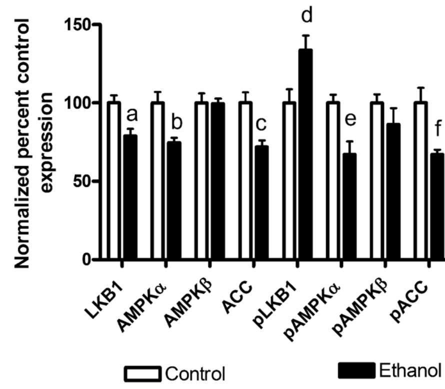
Figure 3

C



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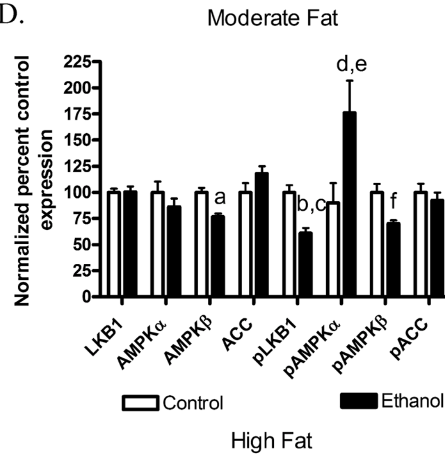

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Figure 3E

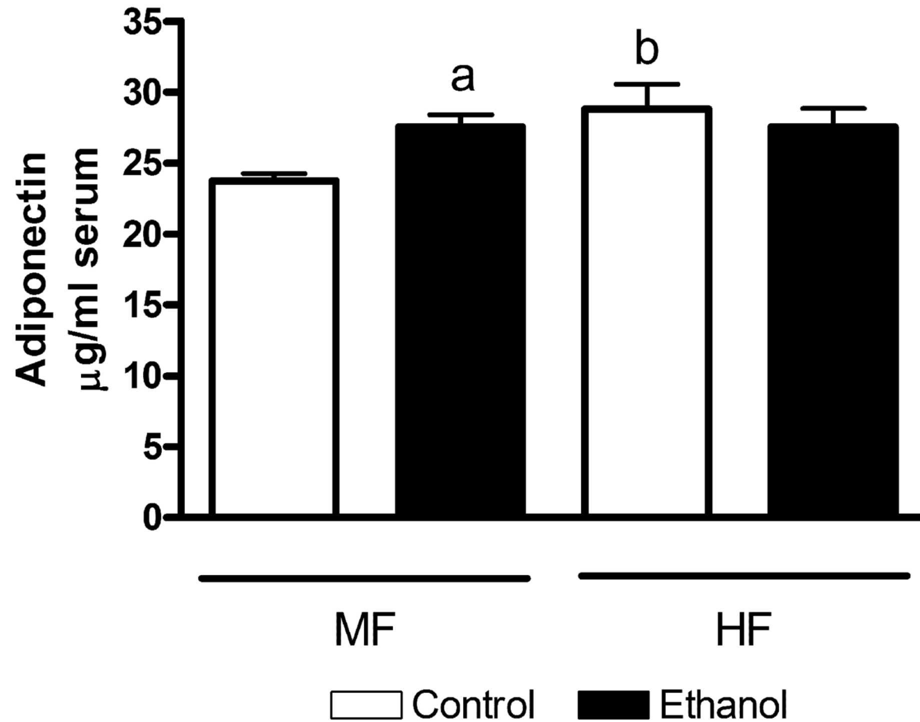

Fig. 3.

Chronic ethanol leads to changes in regulation of the AMPK pathway in MF+E and HF+E mice. Cytosolic fractions from both MF and HF control and ethanol groups were analyzed via standard immunoblotting and probed for total LKB1, AMPKα, AMPKβ, ACC expression and phosphorylation (pSer428 LKB1, pThr172 AMPKα, pSer108AMPKβ, pSer79 ACC) using rabbit polyclonal antibodies (A) MF+E, (B) HF+E, (C) Quantification of the Western blots (actin normalized). (D) Adiponectin levels in moderate fat and high fat ethanol models. Serum isolated from MF and HF control and ethanol groups was analyzed for adiponectin via ELISA. Data are means± SEM as analyzed by two-way ANOVA with a Bonferroni *post hoc* analysis (MF±E compared to HF±E groups). Means without a common superscript letter are significantly different (N=6 mice/group ($p<0.05$)).

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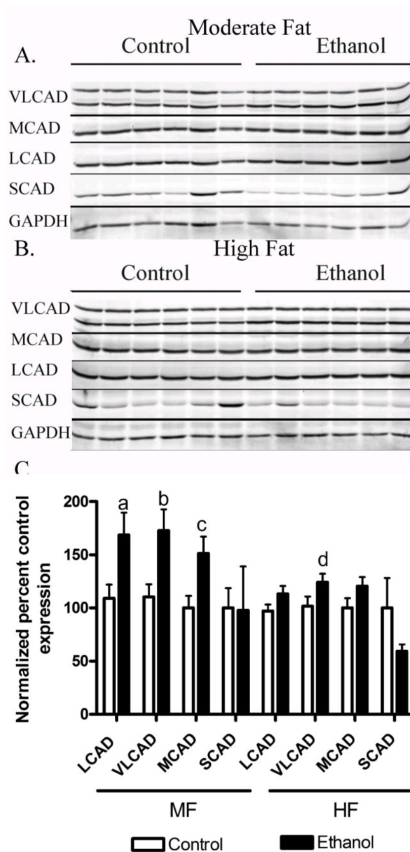

Fig 4.

Effects of increasing dietary fat and ethanol on mitochondrial fatty acyl-CoA dehydrogenase expression. Whole cell lysates from both MF and HF control and ethanol groups were analyzed via standard immunoblotting and probed for VLCAD, LCAD, MCAD and SCAD expression using polyclonal antibodies. (A) MF+E, (B) HF+E, (C) Quantification of the Western blots (GAPDH normalized). Data are means± SEM as analyzed by two-way ANOVA with a Bonferroni *post hoc* analysis (MF±E compared to HF±E groups). Means without a common superscript letter are significantly different (N=6 mice/group ($p<0.05$)).

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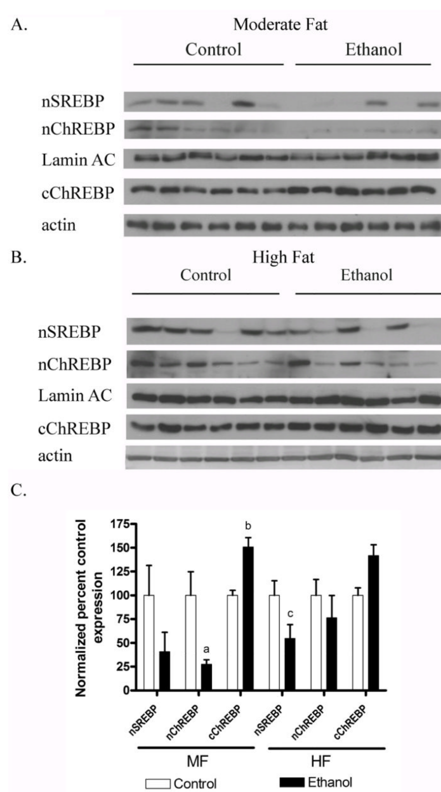

Fig. 5.

Downregulation of nuclear SREBP and ChREBP in MF+E and HF+E mice. Nuclear or cytoplasmic fractions from both MF and HF control and ethanol groups were analyzed via SDS PAGE, Western blotted and probed for nSREBP and nChREBP nuclear localization or cChREBP cytoplasmic localization using rabbit polyclonal antibodies (A) MF+E (B) HF+E. (C) Quantification of the Western blots (actin or lamin AC normalized). Data are means± SEM as analyzed by two-way ANOVA with a Bonferroni *post hoc* analysis (MF±E compared to HF±E groups). Means without a common superscript letter are significantly different (N=6 mice/group ($p<0.05$)).

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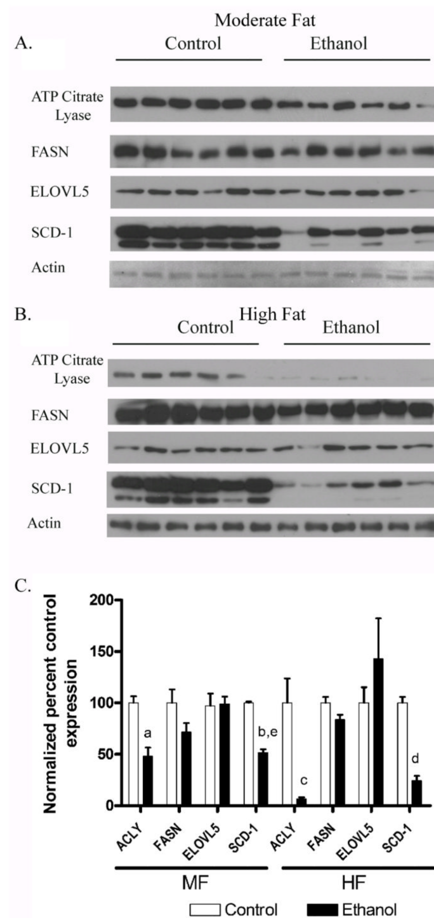

Fig. 6.

Chronic ethanol leads to decreased expression of fatty acid synthetic enzymes in both MF+E and HF+E mice. Cytosolic or microsomal fractions from both MF and HF control and ethanol groups were analyzed via Western blotting and probed for ACLY, FASN, SCD-1 and ELOVL5 (A) MF+E (B) HF+E. (C) Quantification of the Western blots. Data are means ± SEM as analyzed by two-way ANOVA with a Bonferroni *post hoc* analysis (MF±E compared to HF±E groups). Means without a common superscript letter are significantly different (N=6 mice/group ($p<0.05$)). All blots were normalized to actin.

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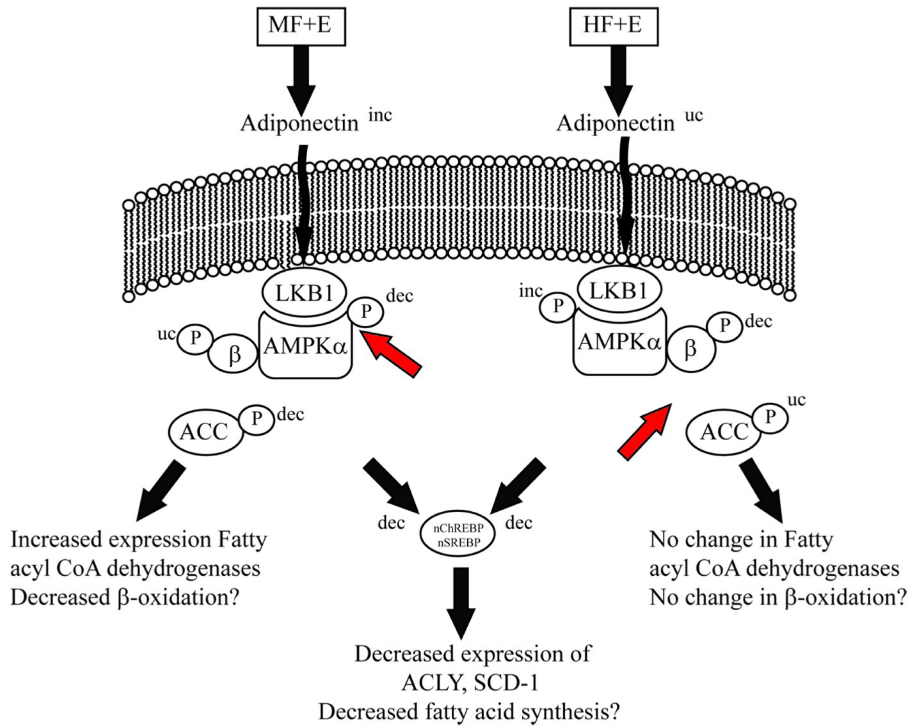

Fig. 7.

Summary of the effects of increased dietary fat plus ethanol on the AMPK pathway and expression of enzymes involved in fatty acid synthesis. In combination with ethanol, a moderate fat diet leads to increased LKB1 phosphorylation, decreased ACC phosphorylation, but no significant change in AMPK phosphorylation. When dietary fat is increased, chronic ethanol consumption decreases LKB1 phosphorylation, increases AMPK phosphorylation but has no significant effect on ACC phosphorylation. In both models, nuclear localization of SREBP1c and ChREBP are decreased corresponding to decreased expression of fatty acid synthetic enzymes. (dec-decreased, inc-increased, uc-unchanged).

Table 1

Diet composition of moderate fat and high fat diets ± ethanol

Diet composition of different groups	MF	MF+E	HF	HF+E
Ethanol (kcal/liter)	-	355	-	355
Protein (kcal/liter)	150	150	150	150
Fat (kcal/liter)	302	302	450	450
C18:2 Linoleic Acid (gram/liter)	17.8	17.8	26.7	26.7
C18:3 Linolenic Acid (gram/liter)	0.4	0.4	0.6	0.6
Saturated Fat (gram/liter)	4.3	4.3	6.4	6.4
monounsaturated fat (gram/liter)	9.2	9.2	13.7	13.7
polyunsaturated fat (gram/liter)	18.2	18.2	27.2	27.2

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Table 2

Physical characteristics of MF+E and HF+E mice.

	Initial Weight	Final Weight	Weight Change	Liver Weight	Percent liver/Body weight	Caloric Intake
30% Fat Control	22.32±0.576[a]	30.03±1.40[a]	7.72±0.957[a]	1.15±0.060[a]	3.85±0.137[a]	16.74±0.174[a]
30% Fat Ethanol	23.32±0.350[a]	27.37±1.161[a]	4.05±1.031[a,b]	1.15±0.031[a]	4.24±0.172[a]	17.38±0.268[a]
45% Fat Control	21.92±1.385[a]	31.02±0.752[a]	9.10±1.237[a]	1.15±0.038[a]	3.78±0.097[a]	17.00±0.205[a]
45% Fat Ethanol	22.92±1.247[a]	25.78±0.647[a]	2.87±1.169[a,b]	1.12±0.036[a]	4.36±0.080[b]	17.78±0.326[b]

Data are means ± SEM as analyzed by two-way ANOVA with a Bonferroni *post hoc* analysis (MF±E compared to HF±E groups). Means without a common superscript letter are significantly different (N=6 mice/group ($p<0.05$)).

Table 3

Biochemical analysis of serum and liver homogenates from MF+E and HF+E mice at the end of the 6 week feeding period. Serum ALT, liver triglycerides and blood ethanol content were determined as described in methods.

	ALT (U/ml)	Triglycerides (rnmol/L/mg tissue)	Blood ethanol mg/dl
30% Fat Control	14.18±1.52[a]	0.18±0.01[a]	
30% Fat Ethanol	26.13±3.28[b]	0.20±0.01[a]	254.4±30.7
45% Fat Control	18.41±4.61[a]	0.15±0.02[a]	
45% Fat Ethanol	42.99±4.52[b]	0.24±0.03[b]	245.4±33.8

Data are means± SEM as analyzed by two-way ANOVA with a Bonferroni *post hoc* analysis (MF±E compared to HF±E groups). Means without a common superscript are significantly different (N=6 mice/group ($p<0.05$)).

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